Supplementary Data

Supplementary Tables

Yeast strains used in this study.

name	alias	genotype	Source and reference
YPH499		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	Sikorski & Hieter (1989)
YPH500		*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	Sikorski & Hieter (1989)
MHY500		*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*	Chen & Hochstrasser (1993)
MHY3110		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 PRE1::YIplac211-PRE1-FH(URA3)*	this study
MHY3722		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 nas2Δ::HIS3*	this study
MHY3728		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 nas2Δ::HIS3 rpt4-G106D*	this study
MHY3755	Sc738	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 gal4Δ::HIS3 sug2-13*	Russell & Johnston (2001)
MHY3756	Sc748	*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 gal4Δ::HIS3*	Russell & Johnston (2001)
MHY3774		*MATα ade2 ura3 his3 trp1 leu2-3,112 can1-100 gal4Δ::HIS3 rpt4-G106D*	this study
MHY3936		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rpn4Δ::kanMX4*	this study
MHY3971		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 RPN5::YIplac211-RPN5-FLAG (URA3)*	this study
MHY3975		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rpt4-G106D RPN5::YIplac211-RPN5-FLAG (URA3)*	this study
MHY4009		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rpt4-G106D RPT1::YIplac211-*	this study

		RPT1FH(URA3)	
MHY4273		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpn2*Δ*::HIS3 YCplac22-rpn2-*Δ*N*	this study
MHY4401	des5	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt4-G106D*	this study
MHY4402	des5α	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt4-G106D*	this study
MHY4457		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 RPN2-GST::HIS3MX6*	this study
MHY4464		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt6(cim3-1)*	this study
MHY4465		*MAT*α *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt6(cim3-1)*	this study
MHY4466		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt1(cim5-1)*	this study
MHY4468		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt6(cim3-1) nas2*Δ*::kanMX4*	this study
MHY4470		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt1(cim5-1) nas2*Δ*::kanMX4*	this study
MHY4561	des16	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpn1-821*	Funakoshi et al. (2002)
MHY4570		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 hsm3*Δ*::HIS3*	this study
MHY4576		*MAT***a** *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 hsm3*Δ*::kanMX4*	this study
MHY4611		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 RPT4-3xFLAG::HIS3MX6*	this study
MHY4617		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt4-G106D-3xFLAG::HIS3MX6*	this study
MHY4625		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt4-G106D hsm3*Δ*::HIS3*	this study
MHY4627		*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpn1-821 hsm3*Δ*::HIS3*	this study

MHY4629	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rpt1(cim5-1) hsm3Δ::HIS3*	this study
MHY4631	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 nas2Δ::kanMX4 hsm3Δ::HIS3*	this study
MHY4633	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rpn4Δ::kanMX4 hsm3Δ::HIS3*	this study
MHY4836	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rpt1(cim5-1) HSM3-FLAG::HIS3MX6*	this study
MHY4972	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 rpn14Δ::HIS3*	this study
MHY4977	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 nas6Δ::HIS3*	this study
MHY4980	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rpn14Δ::HIS3*	this study
MHY4983	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rpn14Δ::kanMX4*	this study
MHY4984	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 nas6Δ::HIS3*	this study
MHY5021	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 NAS2-FLAG::HIS3MX6*	this study
MHY5059	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 rpt4-G106D NAS2-FLAG::HIS3MX6*	this study
MHY5061	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 RPN2-GST::HIS3MX6 NAS2-FLAG::HIS3MX6*	this study
MHY5081	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 RPN2-GST::HIS3MX6 NAS2-FLAG::HIS3MX6 RPT4-V5::kanMX6*	this study
MHY5083	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 RPN2-GST::HIS3MX6 NAS2-FLAG::HIS3MX6 RPT5-V5::kanMX6*	this study
MHY5142	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 hsm3Δ::kanMX4 nas6Δ::HIS3*	this study
MHY5145	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 hsm3Δ::kanMX4 rpn14Δ::HIS3*	this study

MHY5170	*MAT*α *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 rpn14*Δ*::HIS3 nas6*Δ*::HIS3 hsm3*Δ*::kanMX4*	this study
MHY5200	*MAT*α *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 nas2*Δ*::HIS3*	this study
MHY5215	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt6(cim3-1) rpn14*Δ*::HIS3*	this study
MHY5226	*MAT*α *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 nas2*Δ*::kanMX4 hsm3*Δ*::kanMX4 nas6*Δ*::HIS3 rpn14*Δ*::HIS3*	this study
MHY5229	*MAT*α *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 nas2*Δ*::kanMX4 rpn14*Δ*::HIS3*	this study
MHY5230	*MAT***a** *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 nas2*Δ*::kanMX4 nas6*Δ*::HIS3*	this study
MHY5232	*MAT*α *his3-?200 leu2-3,112 ura3-52 lys2-801 trp1-1 rpn14*Δ*::HIS3 nas2*Δ*::kanMX4 hsm3*Δ*::kanMX4*	this study
MHY5233	*MAT*α *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 nas6*Δ*::HIS3 nas2*Δ*::kanMX4 hsm3*Δ*::kanMX4*	this study
MHY5243	*MAT*α *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 nas2*Δ*::HIS3 hsm3*Δ*::kanMX4*	this study
MHY5246	*MAT*α *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 nas6*Δ*::HIS3 rpn14*Δ*::HIS3*	this study
MHY5267	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt4-G106D rpn14*Δ*::kanMX4*	this study
MHY5279	*MAT***a** *his3-*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1 rpn14*Δ*::HIS3 nas6*Δ*::HIS3 nas2*Δ*::kanMX4*	this study
MHY5317	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 nas6*Δ*::HIS3 rpn14*Δ*::kanMX4*	this study
MHY5346	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt6(cim3-1) nas6*Δ*::HIS3*	this study
MHY5407	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 rpt4-G106D nas6*Δ*::HIS3*	this study

The *rpn2-*Δ*N* strain MHY4273 was constructed as in Isono et al., 2007. The *PRE1-FH* strain MHY3110 and the *RPT1-FH* strain MHY4009 were constructed as in Verma et al., 2000.

Plasmids used in this study.

Name	Description	Source and Reference
pFL44-CIM3	2µm, *URA3, RPT6*	Ghislain et al. (1993)
pHF0004	CEN, *TRP1, NAS2*	this study
pHF0006	CEN, *URA3, NAS2*	this study
pHF0007	2µM, *TRP1, NAS2*	this study
pHF0008	2µm,*URA3, NAS2*	this study
pHF0009	CEN, *URA3, RPT4*	this study
pHF0039	2µm, *TRP1, GAL1promoter-RPT4 ORF-CYC1terminator*	this study
pHF0064	CEN, *TRP1, rpn2-ΔN*	this study
pHF0087	2µm, *TRP1, HSM3*	this study
pHF0137	2µm, *TRP1, GAL1promoter-RPT4-V5-CYC1terminator*	this study
pHF0144	2µm, *URA3, RPN14*	this study
pHF0156	2µm, *URA3, NAS6*	this study
pHF0165	2µm, *LEU2, GAL1promoter-RPT5-V5-CYC1terminator*	this study
pHF0174	2µm, *URA3, NAS2promoter-NAS2-FLAG-ADH1terminator*	this study
pHF0185	2µm, *TRP1, GAL1promoter-DSK2 ORF*	Funakoshi et al. (2002)
pHF0187	2µm, *URA3, GAL promoter-DSK2 ORF*	Funakoshi et al. (2002)
pHF0188	2µm, *LEU2, GAL1promoter-RPT5 ORF-CYC1terminator*	this study
pRS314	CEN, *TRP1*	Sikorski & Hieter (1989)
pRS316	CEN, *URA3*	Sikorski & Hieter (1989)
pUB-L-lacZ	2µm, *URA3, GAL1promoter-Ub-L-lacZ*	Bachmair et al. (1983)
pUB-M-lacZ	2µm, *URA3, GAL1promoter-Ub-M-lacZ*	Bachmair et al. (1983)

pUB-P-lacZ	2µm, *URA3, GAL1promoter-Ub-P-lacZ*	Bachmair et al. (1983)
YEp24	2µm, *URA3*	Botstein et al. (1979)
Yeplac112	2µm, *TRP1*	Gietz & Sugino (1988)
YEplac195	2µm, *URA3*	Gietz & Sugino (1988)

Tables describing MS/MS sequencing results:

Table S1. Identified peptides from purified complex from *rpt4-G106D RPN5-FLAG*.

Chromosomal locus	Gene name	No. of peptides
YER021W	*RPN3*	12
YDR427W	*RPN9*	9
YPR108W	*RPN7*	8
YDL097C	*RPN6*	7
YFR004W	*RPN11*	6
YDL147W	*RPN5*	5
YFR052W	*RPN12*	5
YOR261C	*RPN8*	4
YDR363W-A	*SEM1*	2
YCR012W	*PGK1**	1

*Assumed to be a contaminant (highly abundant and occasionally seen in purifications of other proteins).

Table S2. Identified peptides of purified complex in *rpt4-G106D-3xFLAG*.

Chromosomal locus	Gene name	No. of peptides
YOR259C	*RPT4*	5
YOR117W	*RPT5*	4
YHR082C	*KSP1**	1
YHR111W	*UBA4**	1

*Assumed to be contaminants (not found in separate purification).

Table S3. Identified peptides of purified complex I in *rpt4-G106D RPT1-FH*.

Chromosomal locus	Gene name	No. of peptides
YOR259C	*CDC48**	5
YOR117W	*RPN1*	4
YHR082C	*RPT2*	1

*Assumed to be a contaminant (highly abundant, occasionally seen in purifications of other proteins, and found by native gel anti-Cdc48 immunoblotting in control cells lacking the Flag tag on Rpt1).

Table S4. Identified peptides of purified complex II in *rpt4-G106D RPT1-FH*.

Chromosomal locus	Gene name	No. of peptides
YOR259C	*RPN1*	11
YOR117W	*RPT2*	5
YHR082C	*HSM3*	3
YHR111W	*RPT1*	2
YDL229W/YNL209W	*SSB1/2**	2

*Assumed to be a contaminant (highly abundant, occasionally seen in purifications of other proteins, and not seen at the same position as complex II in native gel anti-Ssb1/2 immunoblots).

Table S5. Identified peptides of purified complex I in *HSM3-FLAG rpt1(cim5-1)*.

Chromosomal locus	Gene name	No. of peptides
YHR027C	*RPN1*	14
YIL075C	*RPN2*	12
YDL097C	*RPN6*	9
YDL007W	*RPT2*	8

YOR117W	*RPT5*	8
YDR394W	*RPT3*	8
YER021W	*RPN3*	7
YGL048C	*RPT6*	7
YBR272C	*HSM3*	6
YDR427W	*RPN9*	6
YOR259C	*RPT4*	6
YKL145W	*RPT1*	6
YDL147W	*RPN5*	4
YFR004W	*RPN11*	4
YOR261C	*RPN8*	3
YHR200W	*RPN10*	3
YFR052W	*RPN12*	2
YLR421C	*RPN13*	2
YPR108W	*RPN7*	2
YOL086C	*ADH1**	2
YGL123W	*RPS2**	1
YJR123W	*RPS5**	1
YNL302C	*RPS19B**	1
YOL145C	*CTR9**	1
	*Ubiquitin**	1
YLR044C	*PDC1**	1
YNL178W	*RPS3**	1

*Assumed to be contaminants (highly abundant and occasionally seen in purifications of other proteins).

Table S6. Identified peptides of purified complex II in *HSM3-FLAG rpt1(cim5-1)*.

Chromosomal locus	Gene name	No. of peptide
YHR027C	*RPN1*	16
YDL007W	*RPT2*	12
YOR117W	*RPT5*	9
YIL075C	*RPN2*	8
YBR272C	*HSM3*	8
YDR394W	*RPT3*	7
YKL145W	*RPT1*	6
YOR259C	*RPT4*	6
YGL048C	*RPT6*	5
YGR232W	*NAS6*	2
YLR421C	*RPN13*	1
YGL004C	*RPN14*	1
YOL145C	*CTR9**	2

*Assumed to be a contaminant (highly abundant and occasionally seen in purifications of other proteins).

Supplementary Experimental Procedures

Isolation of *NAS2* and identification of *rpt4-G106D*

The *des5* mutant, like the previously described *des* mutants, was temperature-sensitive (ts) for growth (Funakoshi et al., 2002). Transformation of these cells with various low-copy yeast genomic DNA libraries and selection for colonies that could grow at 36˚C led to the repeated identification of the *NAS2* gene. Linkage analysis, however, indicated that *NAS2* was not the gene mutated in the *des5* strain. The chromosomal mutation responsible for *des5* ts growth was therefore identified by genetic mapping (Swanson et al., 2001). The mutation was first assigned to chromosome XV with 2-μm chromosomal integrants. Linkage analysis with *kanMX*-marked alleles from the yeast deletion library showed ts growth closely linked to the *YOR263C* locus. Two proteasome genes, *RPT4* and *RPN8*, are near *YOR263C*, and introduction of these genes on *CEN* plasmids identified *RPT4* as the complementing gene. The *rpt4-G106D* mutation was identified by isolating genomic DNA from *des5* cells, PCR amplification of the *RPT4* locus, and automated DNA sequencing. This mutant allele on a plasmid failed to complement *des5* ts growth.

MS/MS identification of protein complex composition

Protein complexes separated by 4-6% native PAGE and visualized with GelCode Blue (Li et al., 2007) were excised and processed by Midwest Bio Services LLC. Samples were digested in-gel with trypsin, and the eluted peptide mixtures was analyzed by LC-MS/MS in a DECA-XP plus ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (ThermoFinnigan). Mass spectra were searched using TURBOSEQUEST software against the

NIH nr protein database. Matches were retained if they had DeltaCn scores higher than 0.08 as well as Xcor scores higher than 1.5 for +1 charged peptides, higher than 2.0 for +2 charged peptides, and higher than 2.5 for +3 charged peptides.

Protein expression in bacteria and antibody production

Plasmids were transformed into E. coli strain BL21(DE3) for expression, and proteins were purified as described previously (Kusmierczyk et al., 2008). The Nas2, Hsm3, Nas6, and Rpn14 proteins were expressed as GST fusions from pGEX-KG. Terrific Broth without glycerol and KH_2PO_4 but with ampicillin was used for growth (Ausubel et al., 1989). Induction of protein expression was performed by addition of 2% lactose to a 500 ml to 2L culture at OD_{600} of 0.3-0.5 at 24~37˚C with slow shaking. After 6-12 hr of induction, 1mM IPTG was added to enhance the induction. After 6-12 h, the cultures were harvested by centrifugation, and the cell pellets were stored at -80°C. Frozen cell pellets were thawed on ice and resuspended in 40-160 ml of lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 10% glycerol, 0.25% Tween-20, 1mM DTT and 1mM PMSF). The suspensions were lysed by sonication. The resulting total crude lysate was centrifuged at 10,000 x g for 10 min at 4˚C to separate soluble and insoluble material. The soluble material was diluted with lysis buffer to 500 ml and passed through a 4 ml column of glutathione-Sepharose 4B overnight at 4˚C. The columns were washed with 50 ml lysis buffer. For protein elution for primary antigen injections, the yeast proteins were separated from the N-terminal GST moiety while on the column by incubating with 4 ml of TG buffer (25 mM Tris-HCl, pH7.5, 150 mM NaCl, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10% glycerol, 1mM DTT and 50U/ml of thrombin (Calbiochem, 605160)). For proteins used for boosts, the GST fusions were eluted with elution buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1mM DTT and 50 mM

reduced glutathione). Eluates were concentrated by ultrafiltration if the concentration was lower than 1mg/ml. Rabbit antisera were generated at a commercial site (Cocalico Biologicals, PA). Affinity purified antibodies from these antisera were used for immunoblotting.

Gel filtration

Fractionation of cell extracts by gel filtration was performed as described (Velichutina et al, 2004) with modifications. Yeast cell powder was prepared as described in "Nondenaturing gel analyses of yeast extracts." The cell powder was thawed in sample buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM ATP, 5 mM $MgCl_2$). The suspension was centrifuged at 30,000 rpm for 10 min at 2˚C in a TLA 120.2 rotor in an Optima TLX ultracentrifuge. The supernatant was centrifuged again at 41,000 rpm for 30 min at 2˚C in the same rotor. 500 µl of yeast extract (4.5 mg/ml) was fractionated at 4˚C on a Superose-6 HR 10/30 column equilibrated in sample buffer using an ÄKTA FPLC system (GE Healthcare). Fractions (0.6 ml) were eluted at a flow rate of 0.2 ml/min. Six µl of BSA (10 mg/ml) and 6 µl of 2% sodium deoxycholate were added to each fraction and mixed by vortexing. After incubation for 30 min at 4˚C, 68 µl of 100% TCA was added, mixed and incubated at 4˚C for overnight. The precipitate was washed with -20˚C acetone and dried. The precipitates were dissolved in 80 µl of SDS sample buffer, and 10 µl aliquots were used for SDS-PAGE and immunoblotting.

In vitro 26S proteasome assembly

Cells from four yeast strains were transformed with three plasmids (MHY4457 for plasmids pHF0008, pHF0039 and pHF0188; MHY5061 for pHF0174, PHF0008 and pHF0188; MHY5081 for pHF0174, pHF0137 and pHF0188; and MHY5083 for pHF0174, pHF0008 and

pHF0165). These transformants were cultured in 450 ml of synthetic raffinose media (-Ura-Trp-Leu) at 30˚C. Overexpression of Rpt4 and Rpt5 was induced by adding 50 ml of 20% galactose and incubating for 8 h. Cells were pelleted, washed with ice-cold water and frozen in liquid nitrogen. The frozen cells were ground by mortar and pestle to a fine powder, which was stored at -80˚C.

For each cell powder, 100 µl was thawed in 200 µl of extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 5 mM ATP), and the extract was centrifuged to remove cell debris. To deplete GST-Rpn2-tagged 26S proteasomes and RP-derived particles, the supernatant was mixed with 50 µl of glutathione-Sepharose 4B fast flow resin (GE), and rotated for 1 h at 4˚C. The mixture was centrifuged at 3000 rpm for 30 sec, and the supernatant was then mixed with washed FLAG M2 agarose beads (Sigma) and rotated for 1 h at 4˚C. The beads were washed successively with extraction buffer, extraction buffer with 0.2% Triton X-100, extraction buffer, and finally 26S buffer. The bound proteins were eluted in 20 µl of 26S buffer containing 200 µg/ml FLAG peptide. Concentrations of the Rpt4-Rpt5-Nas2-Flag complexes in each eluate was approximately 3.2 µM (estimated by comparison of CBB band intensities of Nas2 and BSA).

Yeast extract from *rpt4-G106D nas2*Δ was prepared as described above under "Nondenaturing gel analyses of yeast extracts" in the Experimental Procedures. Protein concentration was adjusted to 4 mg/ml, and 70 µl of extract was mixed with 20 µl of Flag-Nas2-Rpt1-Rpt2 eluate and 10 µl of 10x ATP regenerating mix (Verma et al., 2000). The mixture was incubated at 24˚C for 30 min. After incubation, the extract was mixed with 20 µl of 26S buffer containing 60 ng/ml xylene cyanol. Native PAGE and immunoblotting were performed as above. Purified Flag-Nas2-Rpt4-Rpt5 complexes could not be clearly resolved by native gel separation

(not shown). Yeast extract from MHY4457 from which Rpn2-GST was depleted was used as a control (Fig. 4D).

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Supplementary Figure Legends

Supplementary Fig. S1. Genetic interactions between *rpt4-G106D* and *nas2Δ*.

A. The *rpt4-G106D nas2Δ* double mutant is strongly growth-impaired at 30˚C. Cells were streaked onto YPD medium and incubated at the indicated temperatures for 3 d.

B. Mutant *rpt4-G106D* cells accumulate high levels of normally short-lived model proteasome substrates Leu-βgal and Ub-Pro-βgal as measured by β-galactosidase activity assays (Miller units). Loss of Nas2 by itself causes no detectable increase, but the *rpt4-G106D nas2Δ* double mutant accumulates higher levels of these substrates than the *rpt4-G106D* single mutant. Met-βgal is a metabolically stable protein whose levels are unaffected by these mutations. Error bars represent standard deviations from the mean of at least three independent transformants.

Supplementary Fig. S2. Gene-specific and allele-specific interactions between *nas2Δ* and high-copy *NAS2* with proteasome mutations.

A. Loss of *NAS2* also weakly suppresses another *rpt4* allele called *sug2-13* (or *crl13*). Growth analysis was done as in Suppl. Fig. S1A.

B. Loss of *NAS2* does not exacerbate growth defects associated with mutations in other RP base subunits (Rpt1 and Rpt6).

C. High-copy *NAS2* suppresses *rpt4-G106D* but not temperature-sensitive alleles of *RPT1* or *RPT6*. Interestingly, high-copy *NAS2* has a very weak but reproducible inhibitory effect on *rpt1* (*cim5-1*) mutant cell growth. One way to interpret this finding is that Nas2 needs to be released from Rpt4-Rpt5 to allow the latter's integration into a base precursor complex, so excess Nas2 in cells where base assembly is already defective would be deleterious.

Supplementary Fig. S3. Validation of MS/MS identification of the free lid and Rpt4-Rpt5 complexes from *rpt4-G106D* cells.

A. Comparison of proteasome assembly defects in *rpn2-ΔN* and *rpt4-G106D* mutants. Left two panels, Suc-LLVY-AMC fluorogenic substrate overlay assays of whole-cell lysates separated by nondenaturing PAGE. The remaining panels show immunoblots against the indicated proteins. The free lid, documented previously in *rpn2-ΔN* cells, migrates similarly to the complex identified as the free lid in the *rpt4-G106D* mutant.

B. The complex identified as Rpt4-Rpt5 was purified on an anti-Flag resin (mutant or WT Rpt4 was 3xFlag-tagged) and resolved by nondenaturing PAGE. The complex contains Rpt5 but not Rpt1 or Rpn1, two other base subunits.

Supplementary Fig. S4. Sequence alignment of *S. cerevisiae* Hsm3 and *H. sapiens* S5b.

The yeast Hsm3 protein sequence is based on a corrected DNA sequence (M.F. and M.H., unpublished), which differs near the 3' end of the ORF from that deposited at the Saccharomyces Genome Database for the reference S288c strain, which is very closely related to the strains used in the current study. Our sequence is identical to the sequence deposited for the human pathogenic *S. cerevisiae* isolate YJM789. Conservative substitutions are indicated by "+".

Supplementary Fig. S5. Genetic interactions of *hsm3Δ* with different proteasomal mutations.

A. Six-fold serial dilutions of yeast strains incubated under the indicated conditions.

B. Growth comparisons of the indicated single and double mutants. Cells were grown on YPD for 3 d.

C. High-copy *HSM3* can suppress the *rpt1ts* (*cim5-1*) temperature-sensitive growth defect and weakly suppresses *rpt4-G106D*. Suppression by high-copy *NAS2* shows the opposite specificity, with no apparent suppression of *cim5-1*.

D. Similar to C except the *rpn1-821* mutant is tested instead *of cim5-1*.

Supplementary Fig. S6. Analysis of multiple mutants involving the four RP base assembly factors.

A. Growth of all four triple mutant combinations analyzed by serial dilution.

B. Native gel immunoblot analysis of proteasome assembly in assembly factor triple and quadruple mutants. Antibodies used recognized the lid (Rpn5), CP (α4), and base (Rpt5, Rpn1).

C. Suppression of *rpt6ts* (*cim3-1*) growth defects by high-copy *NAS6* and *RPN14*.

D. Both *nas6Δ* and *rpn14Δ* exacerbate the temperature-sensitive growth defect associated with *rpt4-G106D*.

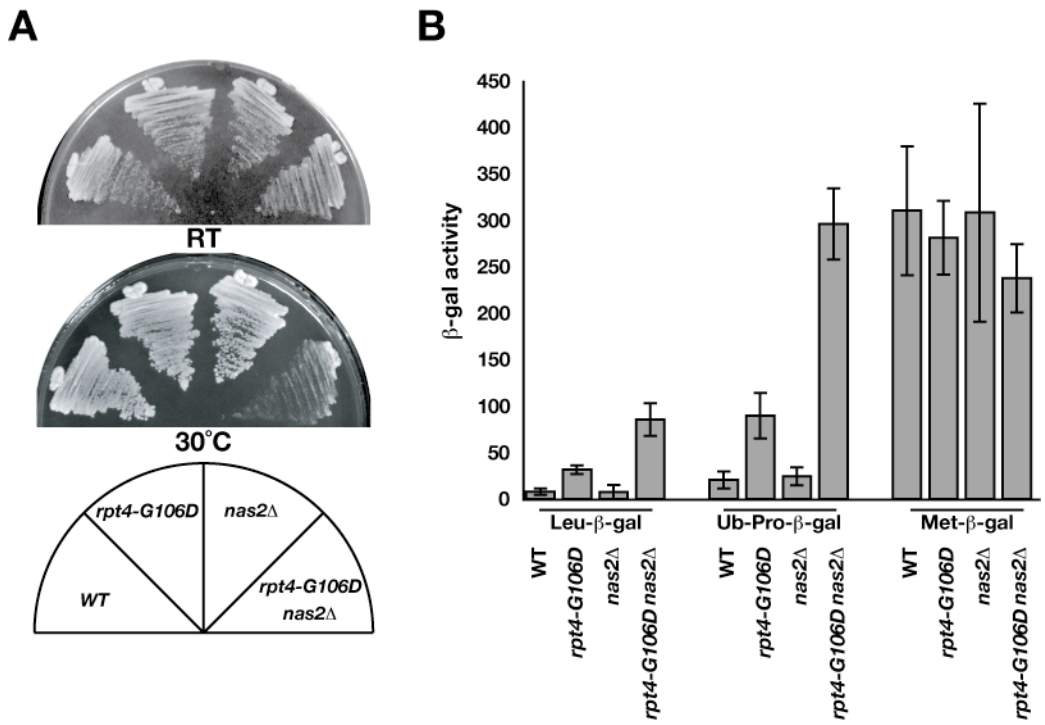
Supplementary Fig. S7. Verification of Nas6 and Rpn14 association with RP precursors.

A. Native gel immunoblot analysis of extracts from the indicated strains demonstrating the presence of Nas6 in RP precursors but not the mature 26S proteasome. Most Nas6 is in a faster migrating species (arrowhead) that is either Nas6 by itself or in a complex with unknown proteins. Similar PAGE immunoblots with anti-Rpt3 (and Rpt6) do not show any reactivity at this position in the gel (not shown). Free base and free lid migrate at similar positions on the gel. Asterisk, crossreacting yeast protein.

B. Immunoprecipitation of Hsm3-Flag in a *cim5-1* mutant verifying the presence of Nas6 in both free base and free RP and Rpn14 in free base but apparently not in the full RP by native gel

immunoblot analysis. In a strain expressing a functional 3xFlag-tagged Rpn14 protein, we also could detect Rpn14 in the free base but not in the full RP (not shown). Anti-Sem1 blotting confirms the presence of Sem1 in Hsm3-associated RP-like complexes even though LC-MS/MS analysis failed to detect any Sem1 peptides. Asterisk, protein aggregate at the top of the separating gel.

C. Confirmation of Rpn14 and Nas6 in Hsm3-Flag-purified complexes using SDS PAGE immunoblot analysis. Substantially more Rpn14 was precipitated from WT cells than from the *cim5-1* mutant even though more free base and free RP are present in the latter strain. This suggests that Rpn14 association with base might be weakened by this mutation in the Rpt1 ATPase. Notably, Nas2 is not co-precipitated in these anti-Flag precipitations.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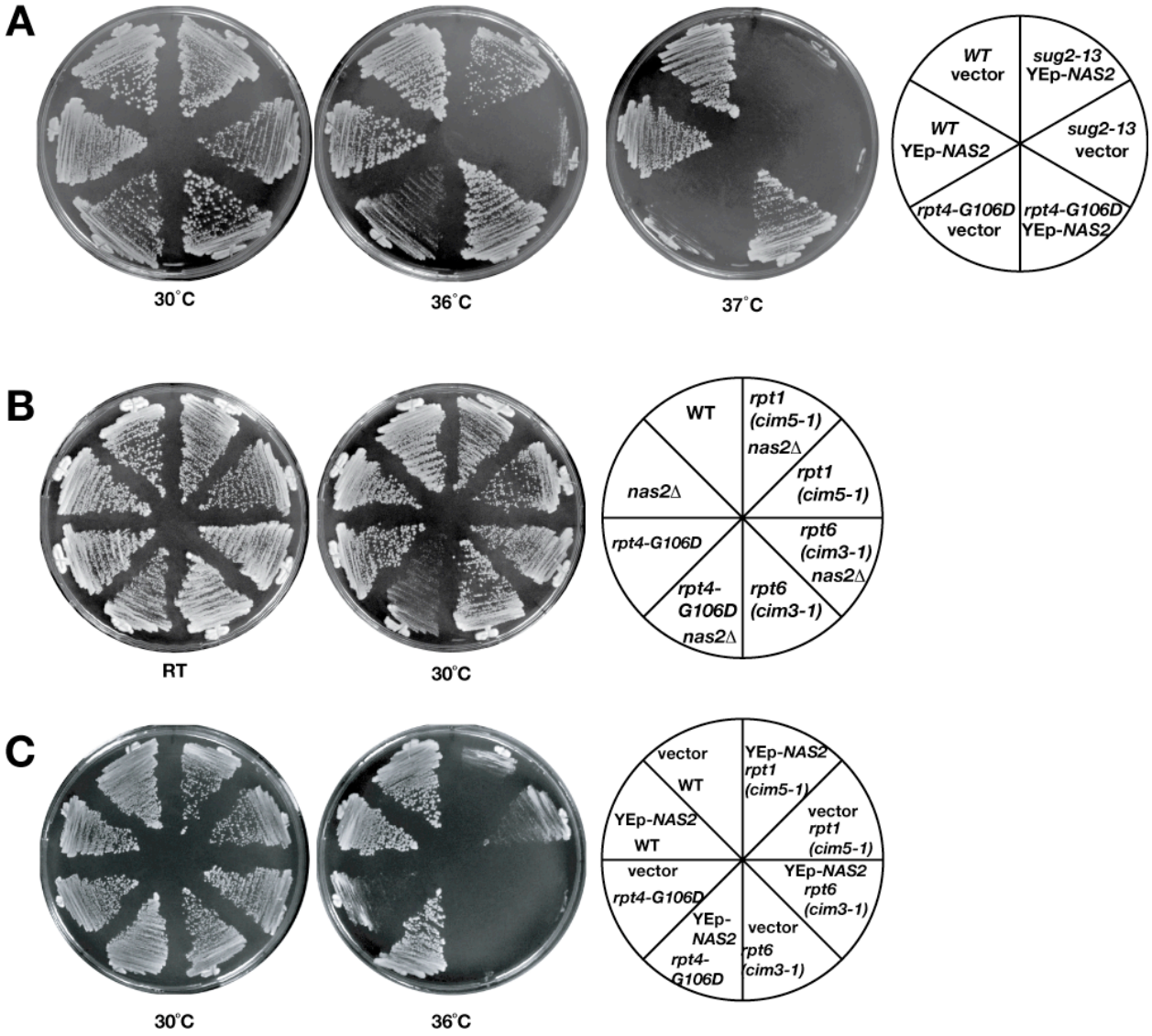
RT
30°C
rpt4-G106D
nas2Δ
WT
rpt4-G106D nas2Δ
B
β-gal activity
450
400
350
300
250
200
150
100
50
0
Leu-β-gal
Ub-Pro-β-gal
Met-β-gal
WT
rpt4-G106D
nas2Δ
rpt4-G106D nas2Δ

Figure S1


A
30˚C
36˚C
37˚C
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sug2-13 YEp-NAS2
WT YEp-NAS2
sug2-13 vector
rpt4-G106D vector
rpt4-G106D YEp-NAS2
B
RT
30˚C
WT
rpt1 (cim5-1) nas2Δ
rpt1 (cim5-1)
nas2Δ
rpt4-G106D
rpt6 (cim3-1) nas2Δ
rpt4- G106D nas2Δ
rpt6 (cim3-1)
C
30˚C
36˚C
vector WT
YEp-NAS2 rpt1 (cim5-1)
YEp-NAS2 WT
vector rpt1 (cim5-1)
vector rpt4-G106D
YEp-NAS2 rpt6 (cim3-1)
YEp- NAS2 rpt4- G106D
vector rpt6 (cim3-1)

Figure S2

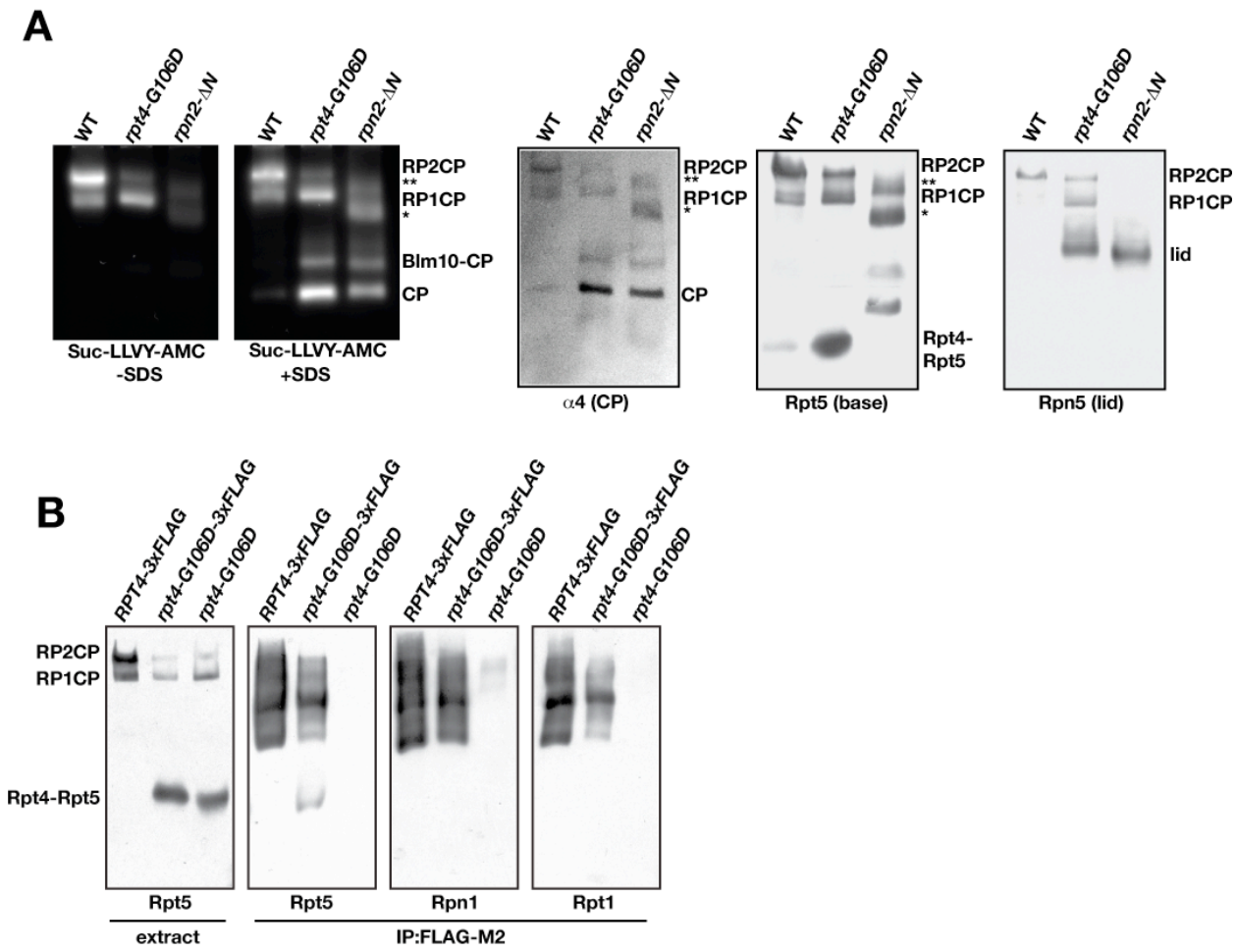
A
WT
rpt4-G106D
rpn2-ΔN
RP2CP
**
RP1CP
*
Blm10-CP
CP
Suc-LLVY-AMC
-SDS
Suc-LLVY-AMC
+SDS
α4 (CP)
Rpt5 (base)
Rpn5 (lid)
Rpt4-
Rpt5
lid
B
RPT4-3xFLAG
rpt4-G106D-3xFLAG
rpt4-G106D
RP2CP
RP1CP
Rpt4-Rpt5
Rpt5
Rpn1
Rpt1
extract
IP:FLAG-M2

Figure S3

```
      UID: Corrected Hsm3 protein vs. Human S5b
      ------------------------------------------------------------------
      Needleman & Wunsch (global)
          Score:   153
       Identity:  22.5%
Hsm3     1 MSEKETNYVENLLTQLENELNEDNLPEDINTLLRKCSLNLVTVVSLPDMDVKPLLATIKR  60
           M+ +    +   + +LE  L E+ L   ++++L+   LN +      ++ + PL + +
 S5b     1 MAAQALALLRE-VARLEAPL-EE-L-RALHSVLQAVPLNELR-QQAAELRLGPLFSLLNE  55

Hsm3    61 FLTSNVSYD-SLNYDYLLDVVDKLVPMA-DFD-DVLE-VYSAEDLVKALRSEIDPLKVAA 116
                 +   S+  + LL  ++  V +A +   D+   +   +D VK L + +   ++
 S5b    56 NHREKTTLCVSI-LERLLQAMEP-VHVARNLRVDLQRGLIHPDDSVKIL-T-LS--QI-G 108

Hsm3   117 CRVIENSQPKGLFATSNIIDILLDILFDEKVENDKLI-TAIEKALERLS-TDELIRRRLF 174
            R++ENS    +    N  ++L  I++    EN  +   AI K+L R+S T   +   LF
 S5b   109 -RIVENSD--AVTEILNNAELLKQIVYCIGGENLSVAKAAI-KSLSRISLTQAGL-EALF 163

Hsm3   175 DNNLPYLVSVKGRMETVSFVRL-IDF-LTIEFQFISGPEFKDIIFCFTK---EEILKSV- 228
           ++NL  L  +K  M+T   VR  + + L IE   +S PE   + +C T     ++L+ +
 S5b   164 ESNL--LDDLKSVMKTNDIVRYRV-YELIIEISSVS-PE--SLNYCTTSGLVTQLLRELT 217

Hsm3   229 -EDILV-F--IELV-NY-YT---K-FLL-E-IRNQ-DKY--WALRH-VKKI-LPVFAQLF 271
            ED+LV    IE+V +  YT   + +L  E + +Q       A         LP F + F
 S5b   218 GEDVLVRATCIEMVTSLAYTHHGRQYLAQEGVIDQISNIIVGADSDPFSSFYLPGFVKFF 277

Hsm3   272 EDTENYPDVRAFSTNC-LLQLFAE-V-SRIE-EDEYSLFKTMDKDSLKIGS--EAKLITE 325
            +     D       C    +F E V   IE +D   +   +D   + +GS  E K + +
 S5b   278 GNLA-VMD-SP-QQICERYPIFVEKVFEMIESQDPTMIGVAVDTVGI-LGSNVEGKQVLQ 333

Hsm3   326 WLELINPQYLVKY-H--KDV-VENYFHVSGYSIGMLRNLSADEECFNAIR-NK--FSAEI 378
                  + L++  H  K+  VE        +I  L  L  +++  + +R  +  FS+ +
 S5b   334 KTGTRFERLLMRIGHQSKNAPVELKIRCLD-AISSLLYLPPEQQTDDLLRMTESWFSS-L 391

Hsm3   379 VLRLPYLEQMQVVETLTRY-E-Y-TS-K-FL-L-NEMPKVMGSLIGD-GSAGAIIDLETV 430
             R P LE  + + +   + E +  + K F  + N+ P     +    G    ++D  +V
 S5b   392 -SRDP-LELFRGISS-QPFPELHCAALKVFTAIANQ-PWAQKLMFNSPGFVEYVVD-RSV 446

Hsm3   431 -HYRNSA-LR-NLLDK-GEEK-LS-VWYEP--L-LREYSKAVNGKNY-STGSETKIADDY 480
            H + S   +  L+      K ++ ++  P  L LR Y  +  G  Y    S T + +
 S5b   447 EHDKASKDAKYELVKALANSKTIAEIFGNPNYLRLRTY-LS-EGPYYVKPVSTTAV-EG- 502

Hsm3   481 VA 482

 S5b   503 AE 504
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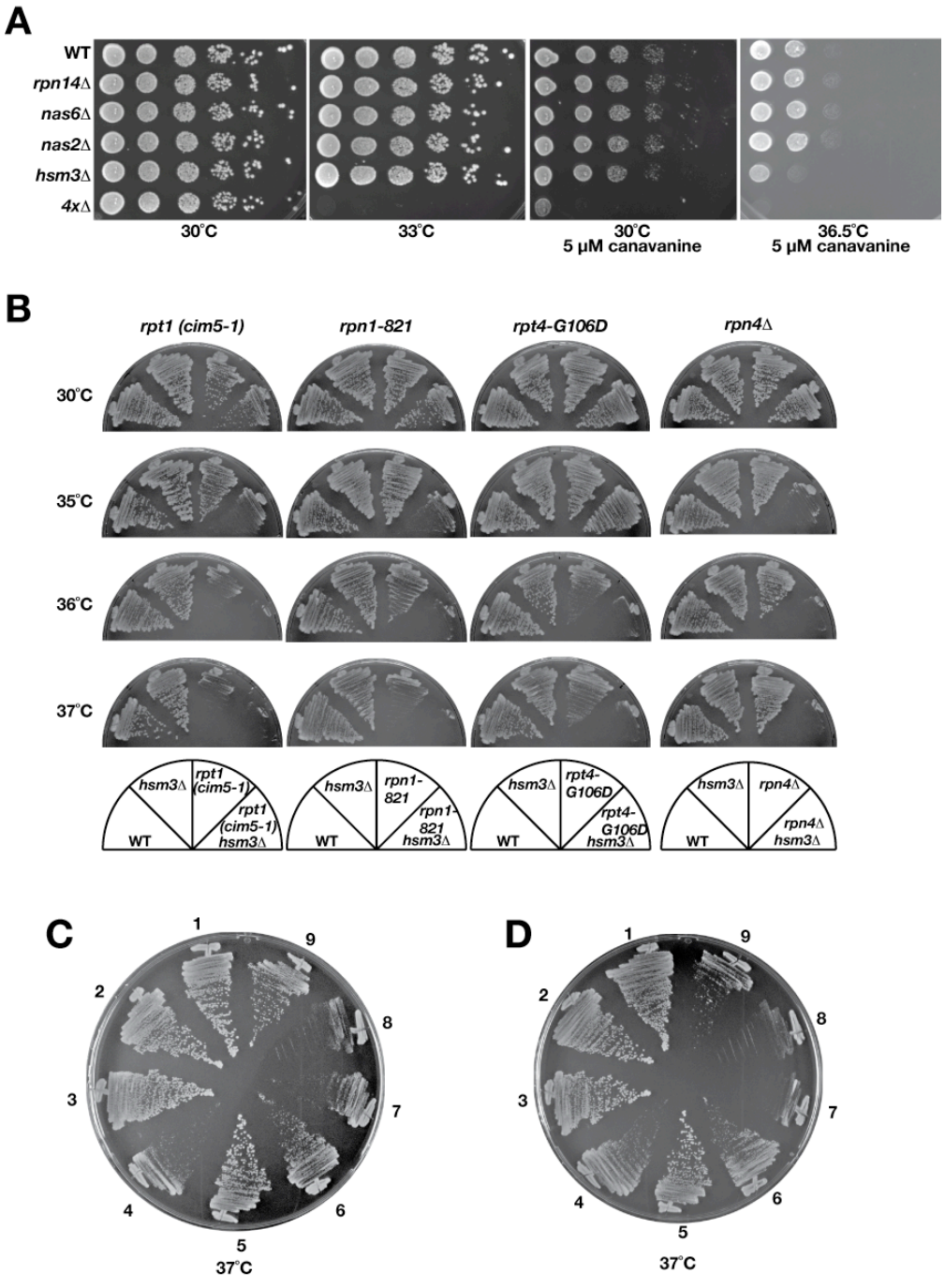
Figure S4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nas6Δ
nas2Δ
hsm3Δ
4xΔ
30°C
33°C
30°C
5 μM canavanine
36.5°C
5 μM canavanine
B
rpt1 (cim5-1)
rpn1-821
rpt4-G106D
rpn4Δ
30°C
35°C
36°C
37°C
hsm3Δ
rpt1 (cim5-1)
rpt1 (cim5-1) hsm3Δ
WT
hsm3Δ
rpn1-821
rpn1-821 hsm3Δ
WT
hsm3Δ
rpt4-G106D
rpt4-G106D hsm3Δ
WT
hsm3Δ
rpn4Δ
rpn4Δ hsm3Δ
WT
C
1
2
3
4
5
6
7
8
9
37°C
1 WT vector
2 WT YEp-NAS2
3 WT YEp-HSM3
4 rpt4-G106D vector
5 rpt4-G106D YEp-NAS2
6 rpt4-G106D YEp-HSM3
7 rpt1(cim5-1) vector
8 rpt1(cim5-1) YEp-NAS2
9 rpt1(cim5-1) YEp-HSM3
D
1
2
3
4
5
6
7
8
9
37°C
1 WT vector
2 WT YEp-NAS2
3 WT YEp-HSM3
4 rpt4-G106D vector
5 rpt4-G106D YEp-NAS2
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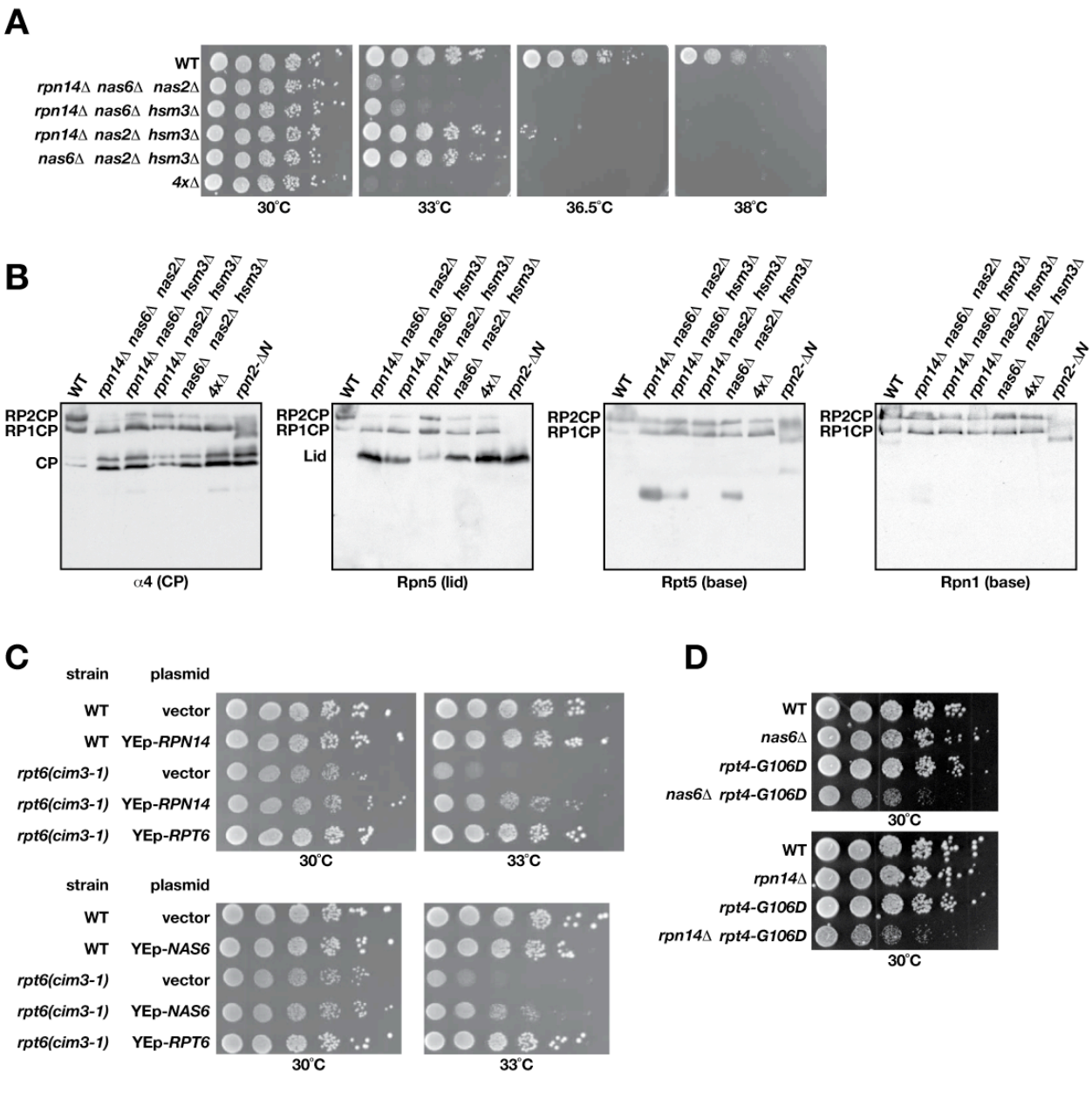
9 rpn1-821 YEp-HSM3

Figure S5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WT
rpn14Δ nas6Δ nas2Δ
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nas6Δ nas2Δ hsm3Δ
4xΔ
30°C
33°C
36.5°C
38°C
B
WT
rpn14Δ nas6Δ nas2Δ
rpn14Δ nas6Δ hsm3Δ
rpn14Δ nas2Δ hsm3Δ
nas6Δ nas2Δ hsm3Δ
4xΔ
rpn2-ΔN
RP2CP
RP1CP
CP
Lid
α4 (CP)
Rpn5 (lid)
Rpt5 (base)
Rpn1 (base)
C
strain
plasmid
WT vector
WT YEp-RPN14
rpt6(cim3-1) vector
rpt6(cim3-1) YEp-RPN14
rpt6(cim3-1) YEp-RPT6
30°C
33°C
WT vector
WT YEp-NAS6
rpt6(cim3-1) vector
rpt6(cim3-1) YEp-NAS6
rpt6(cim3-1) YEp-RPT6
30°C
33°C
D
WT
nas6Δ
rpt4-G106D
nas6Δ rpt4-G106D
30°C
WT
rpn14Δ
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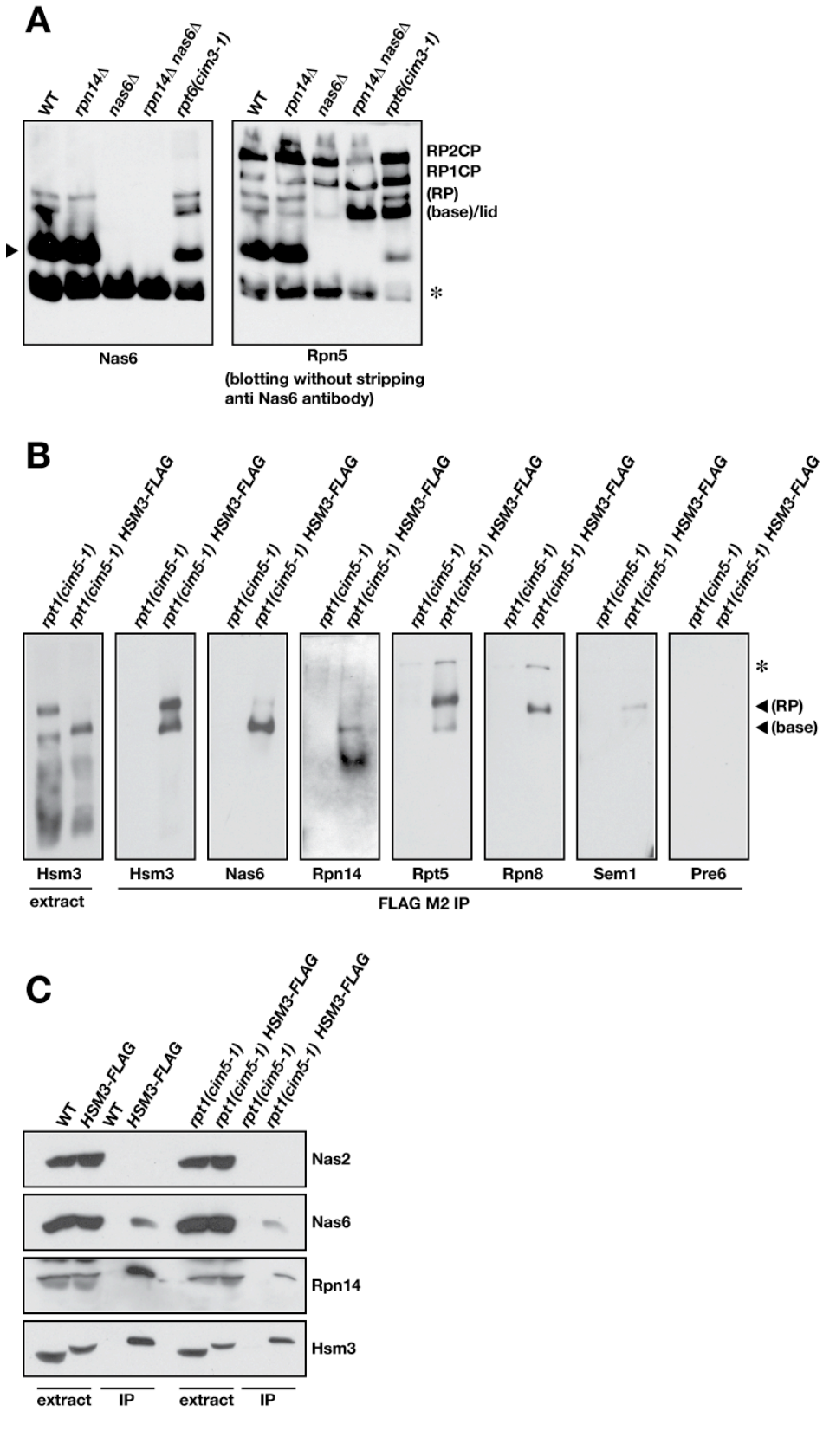
30°C

Figure S6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WT
rpn14Δ
nas6Δ
rpn14Δ nas6Δ
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RP2CP
RP1CP
(RP)
(base)/lid
Nas6
Rpn5
(blotting without stripping
anti Nas6 antibody)
B
rpt1(cim5-1)
rpt1(cim5-1) HSM3-FLAG
(RP)
(base)
Hsm3
extract
Hsm3
Nas6
Rpn14
Rpt5
Rpn8
Sem1
Pre6
FLAG M2 IP
C
WT
HSM3-FLAG
rpt1(cim5-1)
rpt1(cim5-1) HSM3-FLAG
Nas2
Nas6
Rpn14
Hsm3
extract
IP
extract
IP

Figure S7